

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Dugan et al.	Group No.:	1614
Serial No.:	10/083,283	Atty. Docket No.:	53047-31628
Filed:	February 23, 2002		
For:	<i>Carboxyfullerenes and Methods of Use Thereof</i>	Examiner:	Henley III, Raymond J..

DECLARATION OF DR. LAURA L. DUGAN

UNDER 37 C.F.R. §1.132

I, Dr. Laura L. Dugan, declare and state as follows:

1. All of the statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true.
2. I am the first-named inventor of U.S. Patent Application No. 10/083,283 for Carboxyfullerenes and Methods of Use Thereof filed February 23, 2002 ("Patent Application"). I am also a co-inventor on U.S. Patent 6,265,443 for Methods of Treating Neuronal Injury with Carboxyfullerene (Choi et al).
3. My current position is Professor, Larry L. Hillblom Chair in Geriatric Medicine in the Division of General Medicine and Geriatrics at the Department of Medicine, University of California, San Diego.

4. The Patent Application incorporates in it's entirety U.S. Patent 6,265,443 for Methods of Treating Neuronal Injury with Carboxyfullerene (Choi et al). Choi et al shows that $C_{60}(C(COOH)_2)_3$ compounds are effective free radical scavengers (i.e. can eliminate super-oxide radicals) and can reduce neuronal cell death in cultured cells treated with AMPA or NMDA. Choi et al also correctly states that other water soluble $C_{60}(C(COOH)_2)_n$ compounds with n=1 or n=2 are effective free radical scavengers.

5. Prior to the February 23, 2002 filing date of the Patent Application, I demonstrated that the carboxyfullerene derivatives “ C_3 ” ($C_{60}(C(COOH)_2)_3$; malonic acid groups at the *e,e,e* positions), “Penta-1,2” (two stereoisomers of $C_{60}(C(COOH)_2)_2(C(CHCOOH))$; groups at the *e,e,e* positions), “Tetra’s” (four stereoisomers of $C_{60}(C(COOH)_2)(C(CHCOOH))_2$; groups at the *e,e,e* positions), and “ C_3 -lite” (four isomers of $C_{60}(C(CHCOOH))_3$; groups are in the *e,e,e* positions) exhibited comparable superoxide dismutase SOD activity (Exhibit A1 to A3). The demonstration of superoxide dismutase activity of the “ C_3 ”, “Penta”, “Tetra’s” and “ C_3 -lite” derivatives of carboxyfullerene was accomplished using methods that are fully disclosed in the Patent Application. The “ C_3 ”, “Penta”, and “ C_3 -lite” derivatives of carboxyfullerene were also shown to reduce NMDA receptor toxicity, a form of neuronal cell death mediated by mitochondrial superoxide production (Exhibit A4 to A11) prior to the February 23, 2002 filing date of the Patent Application. The demonstration of reduced NMDA receptor toxicity of neuronal cells by the “ C_3 ”, “Penta”, “Tetra’s” and “ C_3 -lite” derivatives of carboxyfullerene was accomplished using methods that are fully disclosed in the Patent Application, which incorporates by reference US Patent No. 6,265, 443 to Choi et al. Since the carboxyfullerene derivatives “ C_3 ”, “Penta”, “Tetra’s” and “ C_3 -lite” all exhibited SOD activity, since “ C_3 ”, “Penta”, and “ C_3 -lite” all reduced NMDA receptor toxicity, and since “ C_3 ” was shown in the Patent Application to increase lifespan when administered to mice, one skilled in the art would have expected that all of these carboxyfullerene derivatives would also have had similar lifespan-extending capabilities as “ C_3 ” at comparable *in vivo* doses. It is therefore my belief that additional carboxyfullerene derivatives other than “ C_3 ” could have been used at the time of February 23, 2002 filing date of the Patent Application to obtain lifespan increases.

5. I further declare that all statements herein made by my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application.

Dr. Laura L. Dugan

July ___, 2005

Characterization of superoxide dismutase (SOD) activity and neuroprotection against NMDA excitotoxicity for the e,e,e series of C₆₀ malonic acid and acetic acid derivatives

Explanation of terms:

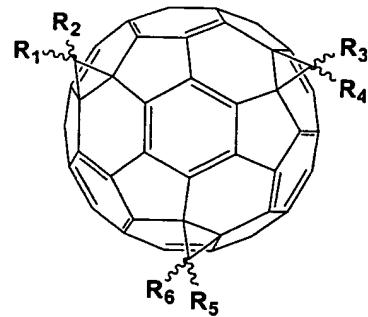
"Hexa" = C₃ (C₆₀(C(COOH)₂)₃ where the malonic acid groups are all at the e,e,e positions (1)

"Penta's" = (C₆₀(C(COOH)₂)₂(C(CHCOOH)) where the groups are at the e,e,e positions. There are two stereoisomers (2)

"Tetra's" = (C₆₀(C(COOH)₂)(C(CHCOOH))₂ where all groups are in the e,e,e positions. There are four stereoisomers (3)

"C₃-lite" = (C₆₀(C(CHCOOH))₃ where all groups are in the e,e,e positions. There are 4 isomers.

1. R₁=R₂=R₃=R₄=R₅=R₆=COOH (C₃)
2. R₁=H, R₂=R₃=R₄=R₅=R₆=COOH
(Penta Pair)
3. R₁=R₃=H, R₂=R₄=R₅=R₆=COOH (Tetra Quartet)
4. R₁=R₃=R₅=H, R₂=R₄=R₆=COOH (C₃-lite)
5. R₁=R₂=COOBu,
R₃=R₄=R₅=R₆=COOMe
6. R₁=R₂=R₃=R₄=COOBu,
R₅=R₆=COOMe
7. R₁=R₂=COOH, R₃=R₄=R₅=R₆=COOMe
8. R₁=R₂=R₃=R₄=COOH, R₅=R₆=COOMe
9. R₁=H, R₂=COOH,
R₃=R₄=R₅=R₆=COOMe
10. R₁=R₃=H, R₂=R₄=COOH,
R₅=R₆=COOMe



EXHIBIT

Al

Method based on S. J. Kim et al., *Bioch. Biotech. Biochem.* LD 946-
~~assay for superoxide scavenging~~ 59 (1995) 822-826.

for Reagents: Tris-cacodylic acid buffer 55.6 mM Tris - 55.6 g.a.,
pH 8.2 - RH. med. = TCB solution

40 mM Pyrogallol in 10 mM HCl. 10 μ l dH₂O + 8.6 μ l
11.6 N HCl + 50.5 mg pyrogallol (m.w 126)

* Tried 2 mM pyr. first \rightarrow no absorbance A.

Reaction: 1.8 ml TCB solution

• 2 ml Pyrogallol solution

• 1 ml SOD (10 U /ml) or 0.1 ml H₂O

2:1 ml

Vortex, pipet rapidly into cuvettes.

Kinetic protocol \Rightarrow 420 nm, RT, blanked against
H₂O. SOD/TCB blank = H₂O absorbance
at 420 nm.

Plan: 2 ml reaction + C₃ dose-response:

0 μ M, 10 μ M, 30 μ M, 100 μ M, 300 μ M, 500 μ M

Lot 99L87R

Add as 0.1 ml solutions.

C₃ = 0.1 ml TCB

10 μ M = x (2.5 mM \times 2.1 ml \times 10 μ M) $x = 0.84 \mu$ l + 99 μ l TCB

30 μ M $x = 2.5 \mu$ l + 97 μ l TCB

100 μ M $x = 8.4 \mu$ l + 92 μ l TCB

300 μ M $x = 25 \mu$ l + 75 μ l TCB

500 μ M $x = 42 \mu$ l + 58 μ l TCB

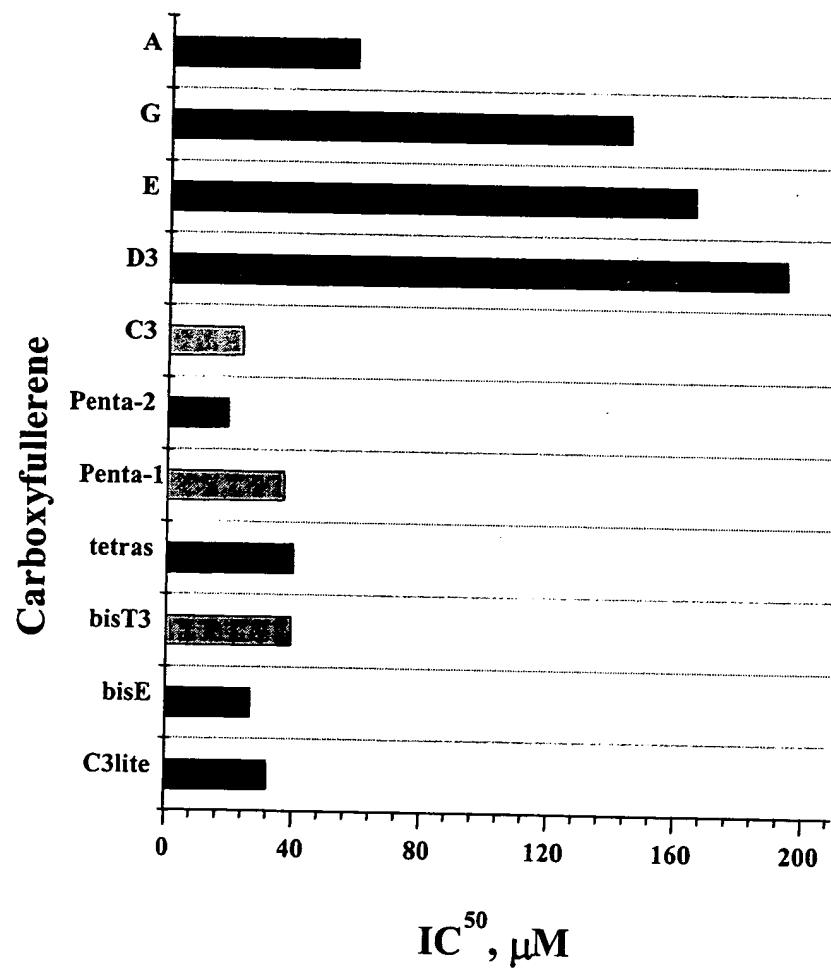
500 μ M \rightarrow absorbance off-scale

Some \downarrow So. Try 20 mM Pyr. and 10 mM Pyr. \pm
some C₃ conc.

10 mM pyr. \rightarrow 2 ml 40 mM Pyr + 6 ml 10 mM HCl

* Need to run + SOD control against 10 mM Pyr.

EXHIBIT



EXHIBIT

A3

and 117 PH.

Plates:

Plan: NMDA 200 μ M $\times 10$ min }
 + Hexa 10 } all 3 pits each
 + " 30 }
 + " 100 }
 + Penta (1) 10 }
 + " 30 } 2nd plate - Penta (2)
 + " 100 }
 BLK }
 by VL

$$\text{Hexa} = 99.5110-1 \quad 9.368 \text{ mM}$$

$$\text{Penta (1)} = 99.5110-3 \quad 9.766 \text{ mM}$$

$$\text{AMPA same plan. } A_{10 \mu\text{m}} \quad \text{Penta (2)} = 99.5110-5 \quad 12.953 \text{ mM}$$

$$\text{Calcs: } (1.0 \text{ ml})(300 \text{ NMDA}) = X 20 \text{ mM} \quad X = 15 \mu\text{l NMDA}$$

$$(1.0 \text{ ml})(15 \text{ Hexa or.}) = X 9.368 \text{ mM} \quad X = 1.6 \mu\text{l Hexa}$$

$$\begin{array}{rcl} 45 & = X 9.368 \\ 150 & & \end{array} \quad X = 4.8 \mu\text{l "}$$

$$\begin{array}{rcl} 150 & & X = 16 \mu\text{l "} \\ & & \end{array}$$

$$\begin{array}{rcl} (15 \text{ Penta (1)}) & = X 9.766 & X = 1.5 \mu\text{l Penta 1} \\ 45 & = X 9.766 & X = 4.6 \mu\text{l "} \\ 150 & = X 9.766 & X = 15 \mu\text{l "} \end{array}$$

$$\begin{array}{rcl} (15 \text{ Penta (2)}) & = X 12.953 & X = 1.2 \mu\text{l Penta 2} \\ 45 & = X 12.953 & X = 3.5 \mu\text{l "} \\ 150 & = X 12.953 & X = 11.5 \mu\text{l "} \end{array}$$

$$\begin{array}{rcl} (1)(\text{AMPA } 15) & = X 10 \text{ mM} & X = 1.5 \mu\text{l AMPA} \\ (1)(\text{MK } 15) & = X 10 \text{ mM} & X = 1.5 \mu\text{l MK} \end{array}$$

$$\begin{array}{rcl} (1)(15 \text{ C}_3) & = X 25 \text{ mM} & X = 0.6 \mu\text{l} \\ (45) & = X 25 & X = 1.8 \\ (150) & = X 25 & X = 6 \mu\text{l} \end{array}$$

2m @ 7:30 PM

EXHIBIT

A4

10/15/99 X300

Conditions:

BLK

AMPA 8 μ M / 10 MK+ (1) 0.5 μ M+ ' 1 μ M+ 3 μ M+ 10 μ M

Same for (3) and (5)

Calcs: 1:10 dilution of compounds \rightarrow in MS(1) Hexa = 937 μ M(3) Pentol = 977 μ M

(5) Penta 2 = 1.295

$$(1.25 \text{ nL})(0.75 \mu\text{M}) = x (937) \quad x = 1 \text{ nL}$$

$$x (977) \quad x = 0.96 \text{ nL}$$

$$x (1.295) \quad x = 0.72 \text{ nL}$$

$$(1.25 \text{ nL})(1.5) = x \text{ above} \quad x = 2 \text{ nL}$$

$$x = 1.92 \mu\text{L}$$

$$x = 1.44 \text{ nL}$$

$$(1.25 \text{ nL})(4.5 \mu\text{M}) = x \text{ above} \quad x = 6 \text{ nL}$$

$$x = 5.8 \text{ nL}$$

$$x = 4.3 \text{ nL}$$

$$(1.25 \text{ nL})(15 \mu\text{M}) = x \text{ above} \quad x = 2 \text{ nL}$$

order:

$$x = 1.92 \text{ nL}$$

use basic
stocks

$$x = 1.44 \text{ nL}$$

$$\text{AMPA: } (1.25 \text{ nL})(12 \mu\text{M}) = x 10 \text{ mM} \quad x = 1.5 \text{ nL}$$

$$\text{MK: } (1.25 \text{ nL})(15 \mu\text{M}) = x 10 \text{ mM} \quad x = 1.875 \text{ nL}$$

In @ 5:30 PM.

EXHIBIT

Condi:

BLK

NMDA 200 μ M \times 10 min+ Hexa 3 μ M

" 10

" 30

" 100

same for pensta-1, pensta-2

Calcs:

$$(1.25 \text{ ml}) (300 \mu\text{M} \text{ NMDA}) = x \text{ 20 mM} \quad x = 18.75$$

$$(1.25 \text{ ml}) (4.5) = x \text{ 93.7 } \mu\text{M}$$

$$97.7 \mu\text{M}$$

$$1.295 \text{ mM}$$

$$x_H = 6 \mu\text{L}$$

$$x_{P1} = 5.8 \mu\text{L}$$

$$x_{P2} = 4.3 \mu\text{L}$$

$$(1.25) (15) = x \text{ 93.68 mM}$$

$$97.76 \mu\text{M}$$

$$1.293 \text{ mM}$$

$$x_H = 2 \mu\text{L}$$

$$x_{P1} = 1.92 \mu\text{L}$$

$$x_{P2} = 1.44 \mu\text{L}$$

$$(1.25) (45) = x \text{ 9.368 mM}$$

$$x (9.766 \mu\text{M})$$

$$x (12.953 \text{ mM})$$

$$x_H = 6 \mu\text{L}$$

$$x_{P1} = 5.8 \mu\text{L}$$

$$x_{P2} = 4.3 \mu\text{L}$$

$$(1.25) (150) = x \text{ same}$$

$$x \text{ same}$$

$$x \text{ same}$$

$$x_H = 20 \mu\text{L}$$

$$x_{P1} = 19.2 \mu\text{L}$$

$$x_{P2} = 14.4 \mu\text{L}$$

TUE (a) 1:30 PM

EXHIBIT

A6

3 Plates: see p. 34 for calculations

for (3) Penta-1 $\rightarrow 100\mu M$ used now
10+ "3b" 11.58mM

$$\times (11.58\text{mM}) = (1.25\text{mL})(150\mu M) \quad x = 16.2\mu L$$

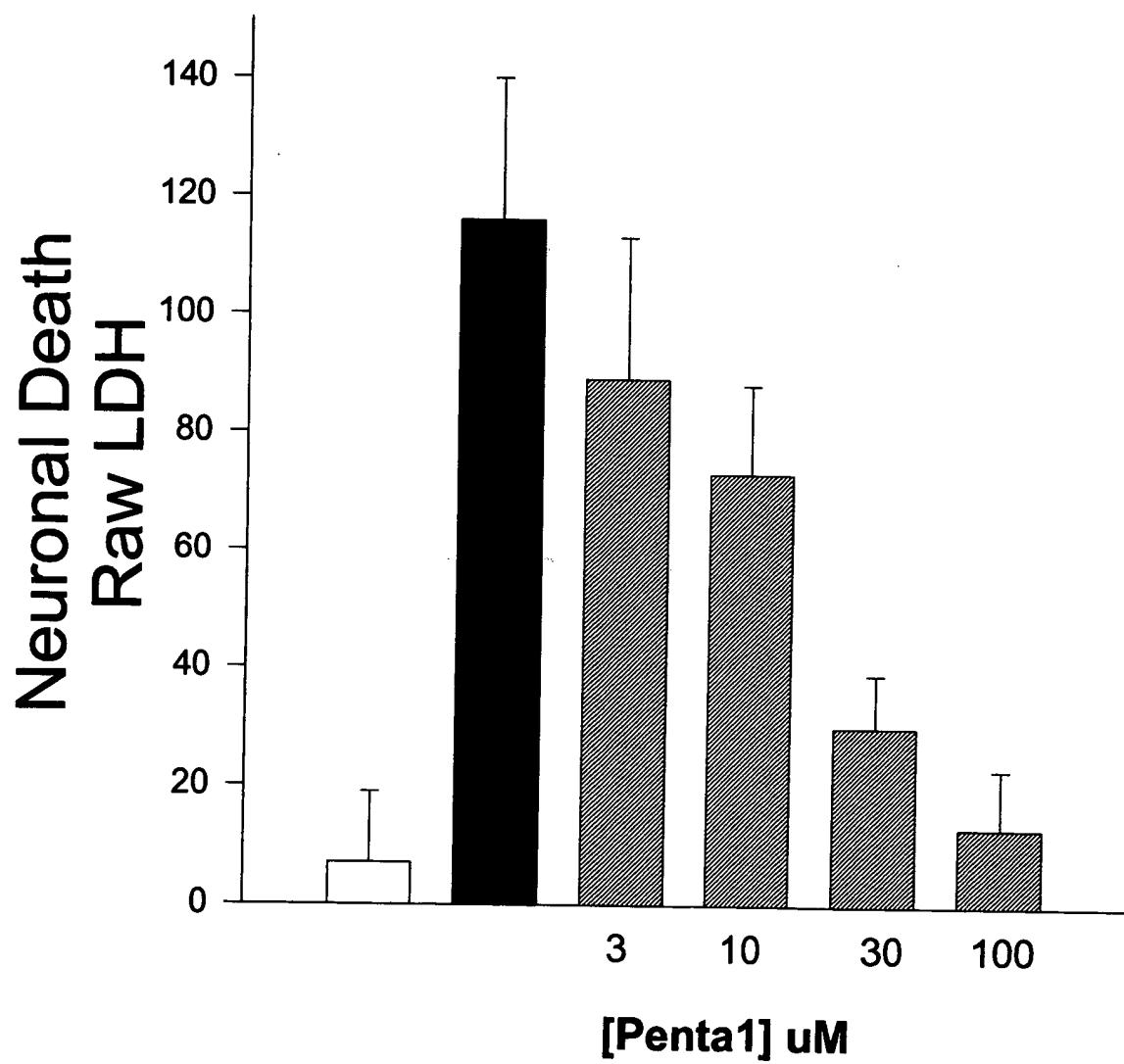
for (5) Penta-2 $\rightarrow 100\mu M$ used now
10+ "5b" 9.43mM

$$\times (9.43\text{mM}) = (1.25\text{mL})(150\mu M) \quad x = 19.9\mu L$$

EXHIBIT

A7

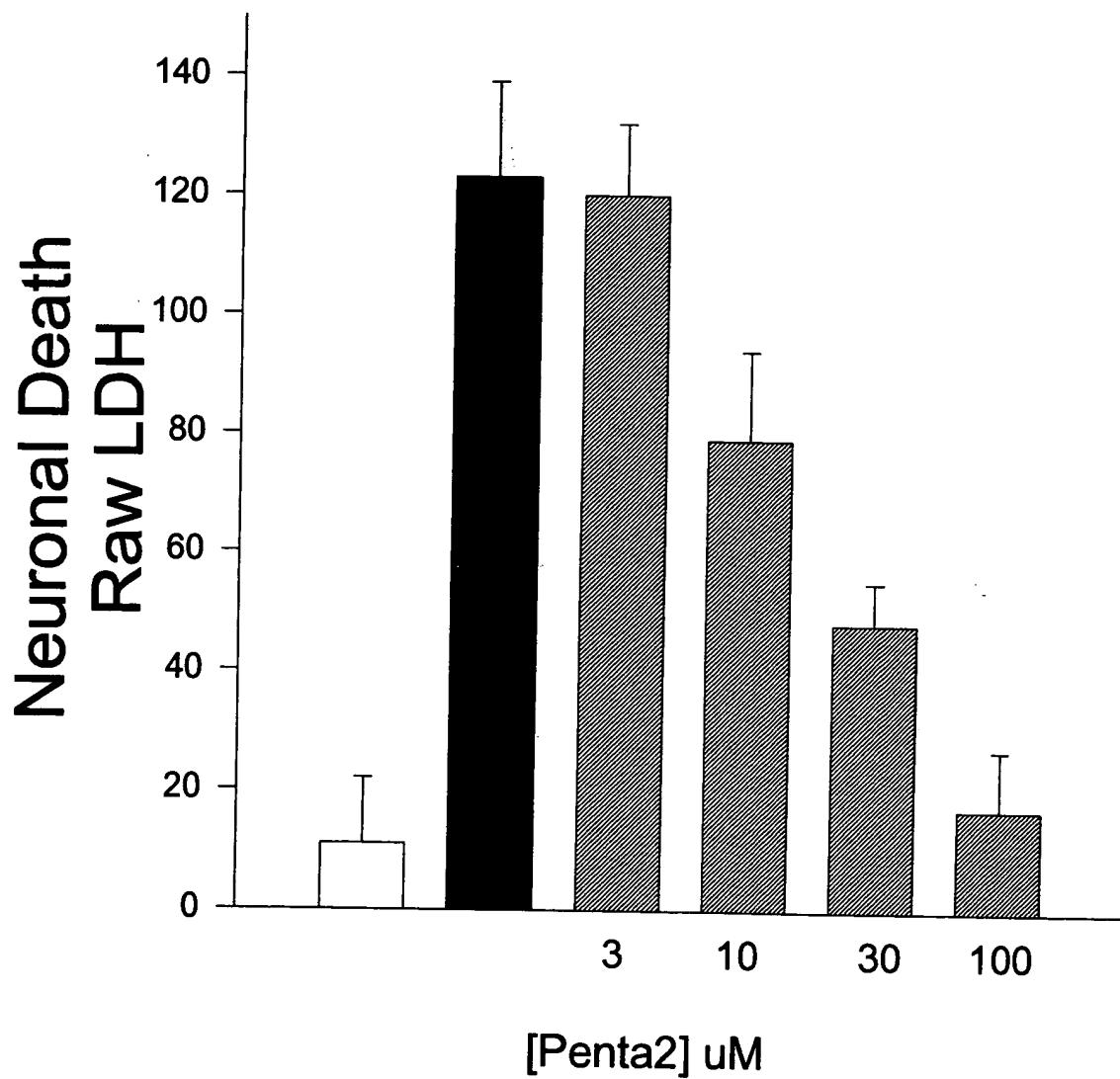
11-14-99
NMDA 200 x 10 min
Penta-1 C₃ (3)



EXHIBIT

A8

11-14-99
NMDA 200 x 10 min
Penta-2 C₃ (5)



EXHIBIT

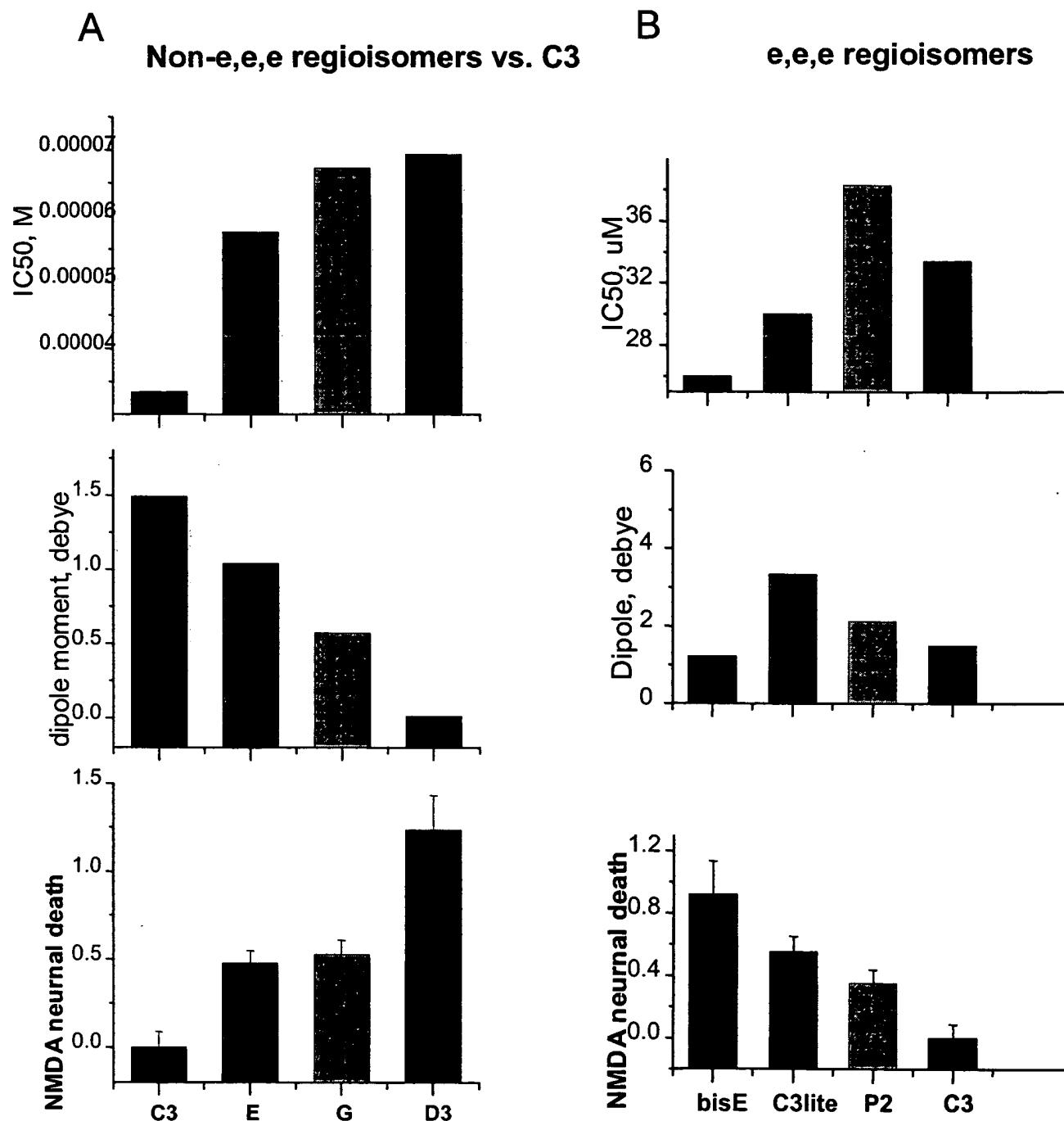
A9

Name	Size	Date	Time	Attr	Type
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PNT1NMDA.SPW	21,294		11/14/1999	8:20 PM	a SPW File
PNT2NMDA.SPW	21,241		11/14/1999	8:19 PM	a SPW File

EXHIBIT

A10

Comparison of various properties of compounds and relationship to neuroprotective efficacy. The IC₅₀ values are for SOD activity, and show that bisE, C3, C3-lite and P2 all have very good SOD activity (note scales are different for graph in A and graph in B).



EXHIBIT